ECOLOGY, BEHAVIOR AND BIONOMICS

Reproductive Behaviour of *Crocidosema* (=*Epinotia*) *aporema* (Walsingham) (Lepidoptera: Tortricidae): Temporal Pattern of Female Calling and Mating

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Edited by José R Trigo – UNICAMP

Neotropical Entomology 39(3):324-329 (2010)

ABSTRACT - *Crocidosema aporema* (Walsingham) is a major pest of legumes in the southern cone of Latin America. The mating behaviour of two allopatric populations (Uruguay and Brazil) of *C. aporema* kept in captivity was studied by observing the posture of calling females, the temporal pattern of pheromone emission and mating, and the response of males to calling females in olfactometer tests. Female calling and mating was observed during the scotophase, from the first to the seventh night after adult emergence. Male response was evaluated at night using a single calling female in a Y-shaped olfactometer. Females adopted a characteristic calling posture, extruding the pheromone gland from the tip of the abdomen. Most females started calling during the second scotophase, and all females called from the third, between the fifth and seventh hours after the onset of the scotophase. Most of the couples mated once throughout the experiment, between the third and sixth night and during the middle of the dark phase. Males preferentially chose the female arm in olfactometer tests, considering both the first arm chosen and the number of visits during the observation period. Our results describe for the first time the temporal pattern associated to the reproductive behaviour of *C. aporema*. We also provide evidence that this tortricid is monoandrous, and that pheromones are used in intersexual communication for mate finding. Our data will be used to optimize the collection of female sex pheromones for chemical characterization in order to develop a monitoring tool for this pest.

KEY WORDS: Chemical communication, sex pheromone, calling periodicity, Y-olfactometer test

Soybean production in southern South America has increased dramatically. In Uruguay, for example, the cultivated area has expanded 30-fold during the period 2000-2006, causing a 10-fold increase in the use of insecticides such as endosulphan and chlorpyrifos (Ferrari 2007). During the same period, the area of soybean in Brazil grew almost 60%, reaching more than 22 million hectares (Conab 2007).

Crocidosema (=*Epinotia*) *aporema* (Walsingham) is a stem and bud borer that has become a major pest of legumes in Uruguay, Argentina, Chile and southern Brazil (Iede & Foerster 1982, Sanchez *et al* 1997, Pereyra & Sanchez 1998, Alzugaray *et al* 1999). *Crocidosema aporema* originated in Costa Rica, and is now widely distributed throughout South America. The general biology and population dynamics of this species is fully described: the larvae undergo five instars, and the entire life cycle takes 33-46 days (at 21-23°C). In temperate regions (31-34°S), the insect is active throughout the year, going through 5-6 generations/year concentrated between November and April (Bentancourt & Scatoni 2006).

The economic importance of *C. aporema* is restricted to the southern cone, due to the year-around availability of hosts (soybean, alfalfa, lotus, peas, flax, red clover) (Biezanko *et al* 1957, Morey 1972, Alzugaray & Ribeiro 2000, Bentancourt & Scatoni 2006). In soybean, the larvae feed on vegetative plant parts affecting the normal growth of the plant. In other legumes, larval feeding can severely affect the flower and prevent the production of seeds, an important commodity in some forage legumes (alfalfa, lotus) (Alzugaray & Ribeiro 2000, Bentancourt & Scatoni 2006).

Insecticides for the control of *C. aporema* are usually employed early in the growing season, causing a strong decline in the populations of natural enemies that would control later-appearing noctuid pests. This underlines the importance of an adequate monitoring system for this species, currently performed by direct observation of the cryptic larvae. The first step in order to develop a pheromone-based monitoring tool for *C. aporema* is the description of its reproductive behavior, specifically the time-frame for female pheromone emission and mating.

Mating in moths usually occurs during a defined period of a day/night cycle, and in most tortricid species it is nocturnal. Sexual activity is largely regulated by the controlled emission of pheromones by the females, which, along with the actual chemistry of the pheromone, is an important factor to ensure reproductive isolation (Roelofs 1995, Raina 1997, Cardé & Haynes 2004).

Here we describe the posture of *C. aporema* calling females, temporal pattern of pheromone emission, courtship and mating behaviour, and the response of males to calling females in olfactometer tests.

Material and Methods

Insects. *Crocidosema aporema* adults were obtained from two laboratory populations that were kept in similar conditions in our laboratories in Brazil and Uruguay. The Brazilian culture was obtained from individuals collected in Fazenda Rio Grande (Parana) and the Uruguayan insects were originally collected in La Estanzuela (Colonia). Both colonies were kept in BOD-type incubators under a 14:10 (L:D) photoperiod, 21-23°C and 60-70% RH. The larvae were reared either in glass tubes or in plastic cups on an artificial diet based on bean homogenate and agar adapted from (Shorey & Hale 1965), by using butter and back beans for the Uruguayan and Brazilian cultures, respectively. Virgin adults were obtained by separating male and female pupae.

Calling behaviour. Virgin females (Uruguayan population, N = 25) were observed during the entire scotophase, from the first to the seventh night after adult emergence. Females were kept individually throughout the experiment in covered, transparent plastic containers (10 cm high, 7 cm diameter). One hour prior to the onset of the scotophase, females were transferred from the incubator to a wooden observation chamber (54 long x 65 cm wide x 76 cm high; 26-28°C and 50-60% RH), which ensured complete darkness during the scotophase. Females were observed every 15 min for their calling behavior under a red light that was lit only during the observations. After the onset of the photophase, females were returned to the incubator.

Mating observations. Upon adult emergence, individual couples (Brazilian population, N = 45) were transferred to transparent plastic containers (7 cm height, 8 cm diameter) covered with mesh and lined in the bottom with moisten filter paper. Courtship and mating were observed under red light, throughout the 10-hour scotophase, during seven consecutive nights. The parameters recorded were the time of mating onset and its length, age of the mating female and the number of mates per couple.

Olfactometer tests. Experiments started 2.5h after the onset of the scotophase, in the same wooden observation chamber used for the observation of the calling behaviour, and under the same ambient and light conditions. The olfactometer was an all-glass Y-maze with equal arms (17 cm long, 3 cm diameter). Virgin males (Uruguayan population, 1-4 days old, N = 38) were placed individually in glass tubes (10 cm long, 3 cm diameter), which were attached to the central arm of the olfactometer. Identical glass tubes were attached to each of the test arms, one holding a single virgin female in calling posture (2-4 days old) and the other empty to serve as control (arms were alternated between tests). Humidified and pre-cleaned (activated charcoal) air was passed through the olfactometer at a total flow of 2.5 L/min. Males and females were placed in the glass tubes 3h prior to conducting the experiments, and the tube holding a calling female was attached to the arm of the olfactometer 30 min before the onset of the observation period. The behaviour of each male was observed continuously during 20 min, recording the first arm chosen and the number of entrances in both arms. Males that did not leave their glass tubes after 12 min were considered as non responsive.

Statistical analysis. The proportion of calling females was analyzed by a two-factor ANOVA in which the effects of female age and hour within the scotophase were evaluated as independent factors (proportion data transformed as $\arcsin\sqrt{p}$). Post-ANOVA pairwise comparisons were done by the Tukey's test ($P < 0.05$), considering the four observations within 1h as replicates for each individual female. The first choice of males in the olfactometer tests was analyzed by the Chi-square test, and the number of entrances to each arm by the Student's t-test for paired samples.

Results

Calling behaviour. The female posture during calling was clearly different from the normal resting position. Calling females elevated their abdomen, curved the abdominal tip downwards, and raised their wings. In most cases it was possible to observe the putative pheromone gland extruded from the tip of the abdomen (Fig 1). No pulsation of the gland or obvious wing fluttering was observed.

Both female age and time into scotophase significantly

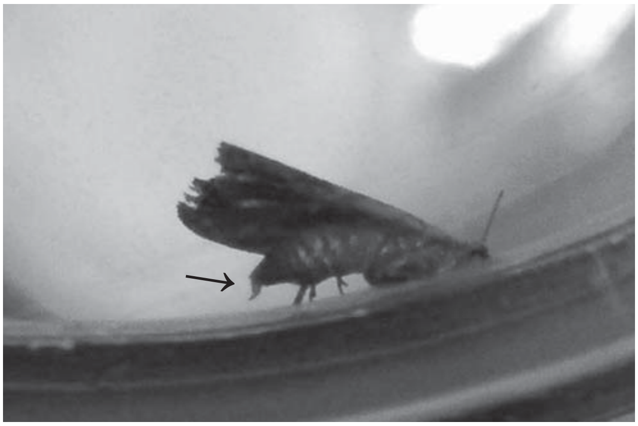

Fig 1 Typical posture of a calling *Crocidosema aporema* female. The arrow indicates the putative pheromone gland protruded from the tip of the abdomen, this structure is only visible when the female is calling (photo taken under red light without flash).

influenced female calling (two-factor ANOVA: F = 339.01, P < 0.001 for age; F = 274.06, P < 0.001 for time into scotophase). Only two females called during their first scotophase, most (88%) of them only begun calling in their second night, and all females called during the three following nights. In the seventh scotophase, 90% of the females called, suggesting the beginning of senescence for the females used in the experiment (Fig 2).

To determine the time period of the scotophase with maximum probability of calling, we averaged the proportion of calling females from those nights that showed no significant differences in calling rates by post-ANOVA pairwise comparisons using the Tukey's test (P > 0.1, nights third through seventh). Females were most likely to call from the fifth through the seventh hour into the scotophase (Tukey's test, P < 0.05 in pair-wise comparisons among scotophase hours). The average time a female called during a single night was 339 ± 25 min (mean ± SEM, N = 25, range 75-585 min), as calculated from the data of the fifth night.

Mating observations. The first mating was observed between the third and sixty scotophase for 82% of the couples, and no matings occurred during the first night. Within the scotophase, most couples (73%) initiated the copula between the fourth and the sixty hour (Fig 3).

Prior to mating, a sequence of events was observed in which the quiescent male responded to the calling female by approaching her while fanning his wings in the upright position, male and female then made contact with their antennae, and immediately turned to engage in mating (Fig 4). Mating lasted an average 81 ± 4 min (mean ± SEM, N = 22, range 50-120 min), and only three couples mated a second time.

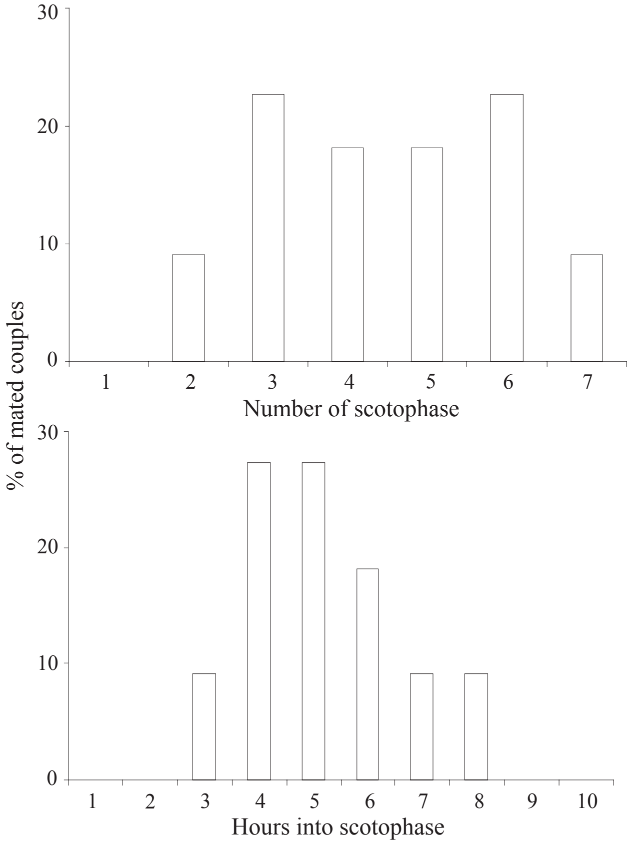

Fig 3 Temporal distribution of mating in *Crocidosema aporema* (N = 22 couples). Bars show the percent of couples that mated for the first time according to: top: number of scotophase after adult emergence; bottom: hour of mating onset within any scotophase.

Response of males in olfactometer. Half of the males did not move from their original tubes, and were not taken into consideration. Those that did respond (N = 19) mostly walked through the olfactometer, taking the wing for

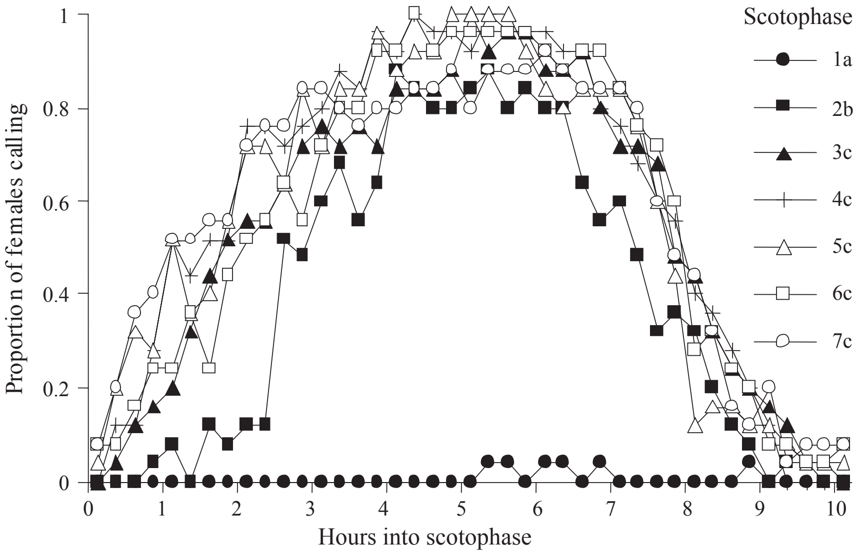

Fig 2 Proportion of females calling during seven consecutive scotophases (10h) following adult emergence. The scotophase number (age of the females) is shown in the legend within the figure. Different letters as superscripts to the scotophase number indicate significant differences in post-ANOVA pairwise comparisons (N = 25; P < 0.05; Tukey´s test).

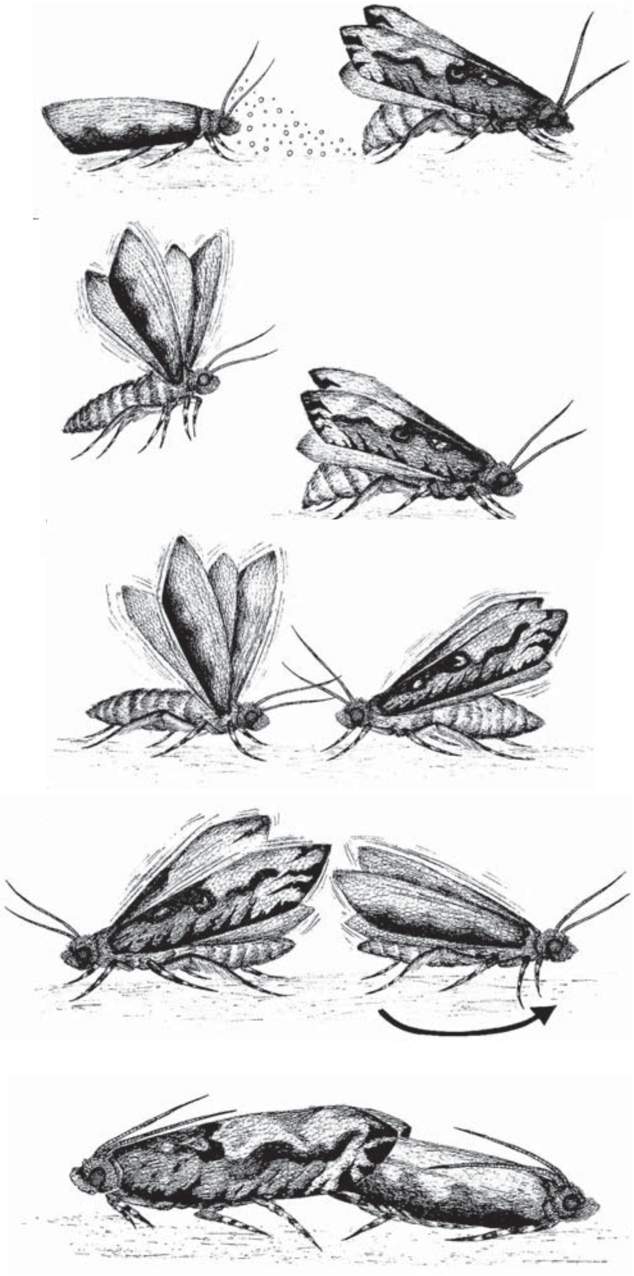

Fig 4 Ethogram of *Crocidosema aporema* (see main text for explanation). Drawings are based on photographs (calling) and direct observations (courtship).

short distances. The males moved considerably within the olfactometer during the 20-min observation period, entering several times in both arms. No courtship behaviour was observed when the males reached the mesh that separated the olfactometer from the tube holding the calling female. A significant majority of males reached the female arm first (74%, Chi Square test: $\chi^2 = 4.26$, $P < 0.04$). Considering the number of entrances to the calling female and control arms, the males preferred the former, entering an average of 3.5 ± 0.8 times to the female arm (range 0-13) and 1.5 ± 0.5 to the control arm (range 0-9) (paired t-test: $t = 2.19$, $P < 0.05$) (Fig 5).

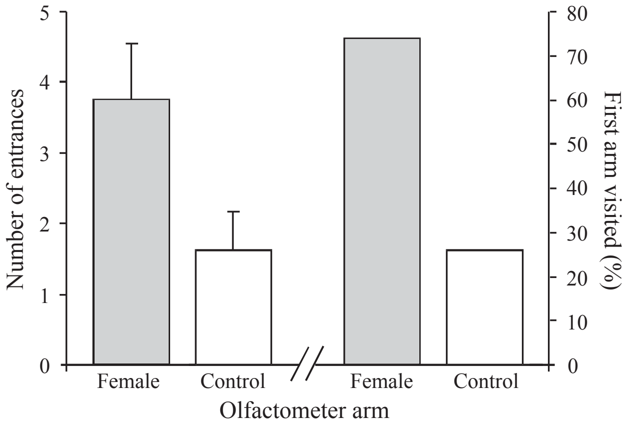

Fig 5 Response of individual males in a Y-maze olfactometer with a choice between a calling female and clean air (N = 19 males). Left: average number of entrances of a single male in each arm, throughout the 20-min observation period (bars indicate standard deviation); right: percent of males that visited first the female or control arms.

Discussion

Pheromone emission in female moths usually involves the extrusion, often rhythmically, of the pheromone gland at the tip of the abdomen, and a stereotyped calling posture (Cardé & Haynes 2004). In the case of *C. aporema*, calling behavior takes place with an immobile posture throughout the calling period. This posture is characterized by the raised wings, the abdomen curved down and the gland continuously exposed.

Our results show that as in most moths (Cardé & Haynes 2004), the sexual activity of *C. aporema* is regulated by the photoperiod. Both males and females increase their flight activity upon the onset of the scotophase, and quickly return to quiescence at the beginning of the photophase. Mating occurs at night, and it is tightly correlated in time with the emission of pheromones by virgin females. Males, in turn, display a typical wing fanning response in the presence of a calling female, and are attracted to the females in a Y-tube olfactometer, indicating the presence of volatile pheromones. The behavioural sequence observed during courtship was not unusual for a moth. Indeed, courtship in *C. aporema* included behavioural units similar to those observed in the tortricid moths *Choristoneura rosaceana* (Harris) and *Pandemis pyrusana* Kearfott (Curkovic *et al* 2006). It may be speculated that behaviors such as the antennal contact prior to both adults turning and engaging in mating may have communication value, such as species recognition or female receptivity cues.

Moths show variation with respect to the frequency of mating and the onset of sexual activity after adult emergence. Such variation may respond to various predictable ecological conditions, to which different species are adapted. For instance, species with a delay in the onset of reproductive behaviour may need to migrate prior to mating in response to habitat deterioration (McNeil *et al* 1997). While two related tortricids, *P. pyrusana* and *Argyrotaenia citrana* (Fernald), are sexually active from their first night after adult emergence (Knight 1996, Knight & Turner 1998), our results show that calling and mating in *C. aporema* was completely absent during the first night. However, most virgin females called within 48h of age, and mating soon followed, suggesting that *C. aporema* does not migrate upon eclosion. Rather, as it has been shown for other tortricids (Jiménez-Pérez *et al* 2002), this short delay may be due to physiological constrains related to ovarian development, rather than to a programmed search for mating or oviposition sites. Engaging in reproduction as early as possible is likely to be advantageous for moths with short adult lives such as *C. aporema* (10 to 25 days in the laboratory; Paula Altesor, unpublished data). Likewise, the probability of mating is expected to increase as the calling period within the scotophase is lengthened. Interestingly, our data suggest that older females tend to begin calling earlier in the scotophase, thereby increasing the calling period. The same tendency has been observed in related and unrelated moth species (Knight 1996, Knight & Turner 1998, Zarbin *et al* 2007), and it is likely to be a more generalized trend among moths.

The frequency of mating can also vary among closely related species, which may be the result of different ecologically-driven balances between direct or genetic benefits obtained by the females at mating, and the various costs associated with it, such as predation or the transmission of diseases (Drummond 1984). Our results show that even in the constant presence of a male during several nights, mated females did not tend to re-mate, which differs from other tortricid females that mate repeatedly (Knight 2007). While we did not attempt to offer new males to the females throughout the experiment, our data suggest that *C. aporema* is a monoandrous species.

Our basic findings concerning the mating behaviour of *C. aporema* may find various uses in the development of novel management methods for this pest, specifically towards the development of a much needed monitoring tool. Indeed, the description of the time pattern of pheromone emission will facilitate the collection and chemical characterization of the sex pheromones by dissections of the glands during the peak of female calling (5-7h into the scotophase).

Finally, it is noteworthy that these studies were conducted independently with two populations from distant locations. In these populations we found the same temporal pattern for female calling (Uruguayan population) and mating (Brazilian population), suggesting that no inter-population variation occurs in this species regarding the time frame of reproductive behavior.

Acknowledgments

The authors wish to thank financial support by CONICYT (Uruguay), INIA (Uruguay), The Lindbergh Foundation (USA), IFS (Sweden), OPCW (multilateral), CNPq-ProSul (Brazil) and Fundação Araucaria (Brazil).

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Received 23/X/08. Accepted 05/V/09.